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Abstract: To investigate the pathogenic role of enteroaggregative Escherichia coli (EAggEC) among human immunodeficiency virus—infected persons, 111 outpatients with and 68 without diarrhea were evaluated. Examination of stool samples included the HeLa cell adherence assay and an EAggEC polymerase chain reaction (PCR) assay using primers complementary for the plasmid locus CVD432. The pCVD432 genotype, adherence phenotype, and patient characteristics were correlated with occurrence of diarrhea by multivariate analyses. EAggEC PCR and adherence assays were positive in 7 (6%) and 24 (22%) patients with diarrhea and in 1 (1%) and 21 (31%) asymptomatic control patients, respectively. Clinical manifestations associated with EAggEC PCR-positive isolates were nonspecific; EAggEC infections were independent of CD4 lymphocyte counts. Of the pCVD432 genotype, 5 (71%) of 7 were resistant to cotrimoxazole and ampicillin, and 1 strain was resistant to ciprofloxacin. Overall, pCVD432 PCR-positive E. coli was the most prevalent intestinal organism associated with diarrhea. The adherence assay results did not correlate with diarrhea

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CONCISE COMMUNICATION

Intestinal Infection Due to Enteroaggregative *Escherichia coli* among Human Immunodeficiency Virus–Infected Persons

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To investigate the pathogenic role of enteroaggregative *Escherichia coli* (EAggEC) among human immunodeficiency virus–infected persons, 111 outpatients with and 68 without diarrhea were evaluated. Examination of stool samples included the HeLa cell adherence assay and an EAggEC polymerase chain reaction (PCR) assay using primers complementary for the plasmid locus CVD432. The pCVD432 genotype, adherence phenotype, and patient characteristics were correlated with occurrence of diarrhea by multivariate analyses. EAggEC PCR and adherence assays were positive in 7 (6%) and 24 (22%) patients with diarrhea and in 1 (1%) and 21 (31%) asymptomatic control patients, respectively. Clinical manifestations associated with EAggEC PCR-positive isolates were nonspecific; EAggEC infections were independent of CD4 lymphocyte counts. Of the pCVD432 genotype, 5 (71%) of 7 were resistant to cotrimoxazole and ampicillin, and 1 strain was resistant to ciprofloxacin. Overall, pCVD432 PCR-positive *E. coli* was the most prevalent intestinal organism associated with diarrhea. The adherence assay results did not correlate with diarrhea.

Human immunodeficiency virus (HIV)–associated diarrhea frequently remains unexplained, despite comprehensive evaluation, including examination of stool samples and endoscopy [1]. An increasing proportion of patients may suffer from intestinal adverse effects of antiretroviral drugs, but the current diagnostic approach may fail to detect relevant and possibly treatable enteric pathogens. Enteroaggregative *Escherichia coli* (EAggEC) are associated with acute or persistent diarrhea among children in tropical and nontropical countries [2, 3] and with foodborne outbreaks, nosocomial infections, and traveler's diarrhea [4]. Recently, EAggEC have been considered as causes of HIV-associated diarrhea [5, 6]. In a study, EAggEC were found more frequently in stool samples of HIV-infected adults with diarrhea than in asymptomatic control patients and appeared to be more often associated with intestinal disease when immunodeficiency was more advanced [6]. Moreover, eradication of EAggEC in HIV-infected patients with otherwise unexplained chronic diarrhea led to resolution or improvement of symptoms in ~80% of treated patients [7].

No widely accepted diagnostic methods to identify EAggEC are available for the routine clinical laboratory. EAggEC is defined by the "stacked brick" aggregative adherence pattern in the HEp-2 or HeLa cell adherence assay [4, 8], the most reliable diagnostic means [6]. Molecular probes to detect different presumed virulence factors of EAggEC have been developed, but neither method has enjoyed frequent application, and a comparison of cell culture assays and molecular diagnostics often have considerable discrepant results [2, 6, 9, 10]. This may be explained, in part, by results of phylogenetic analyses that show a significant strain-to-strain heterogeneity and suggest that EAggEC strains comprise a heterogeneous set of pathogens [4]. Differing virulence by EAggEC strain has been postulated, because healthy volunteers who ingested EAggEC did not consistently develop diarrhea [11]. In the present study, we had 4 goals: (1) to evaluate the prevalence of EAggEC among HIV-infected patients with and without diarrhea, (2) to describe the clinical manifestations associated with EAggEC, (3) to compare the EAggEC polymerase chain reaction (PCR) assay (using primers complementary for pCVD432) with the HeLa cell adherence assay, and (4) to determine the antibiotic resistance of EAggEC in this patient group.

Patients and Methods

Study design. We collected stool samples and clinical data for all patients at the University Hospital HIV outpatient clinic (Zurich) who were evaluated for diarrhea (September 1996 to August

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Informed consent was obtained from all patients, and the study was approved by the University Hospital, Zurich, ethics committee.

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1998) [1]. Patients answered a questionnaire on possible risks for diarrhea and were prospectively followed within the Swiss HIV Cohort Study [12]. Control patients were HIV-infected persons who did not have diarrhea for ⩾4 weeks.

Evaluation of diarrhea. Patients with ⩾2 loose or watery stools per day for ⩾3 days underwent a standardized examination of stool samples (table 1) [1]. Stool samples of patients with diarrhea and control patients were tested for verotoxin-producing, enteropathogenic, and enterotoxigenic *E. coli* [13]. Upper endoscopy or ileocolonoscopy was performed, if chronic diarrhea remained unexplained.

EAggEC cell adherence assay. Four biochemically confirmed *E. coli* colonies were assayed by HeLa cell adherence assay [6]: HeLa cells (American Type Culture Collection, Rockville, MD) were suspended in MEM with 10% fetal calf serum (FCS) without antibiotics, seeded in 8-well chamber slides (Lab-Tek; Nunc, Naperville, IL), and were incubated overnight at 37°C with 5% CO_2. *E. coli* strains to be tested were grown overnight at 37°C in 2 mL of trypticase soy broth (Becton Dickinson Microbiology Systems, Cockeysville, MD), were washed, and were resuspended in PBS. The medium in the chamber wells was replaced with 200 μL of MEM with 1% D-mannose without FCS and antibiotics. We added 20 μL of the *E. coli* suspension (0.5 MacFarland U) to chamber wells, which were incubated for 3 h. Subsequently, the slide with the monolayers was washed 4 times with PBS, fixed with methanol, and stained with Giemsa. A search for aggregative adherence pattern was performed in a blinded fashion by 2 investigators.

Molecular identification of EAggEC. Amplification with primers pCVD432/start and pCVD432/stop was performed with bacterial growth from MacConkey agar or with individual *E. coli* colonies, as described elsewhere [10].

Antibiotic susceptibility testing. Disk-diffusion susceptibility testing was performed on Mueller-Hinton agar plates, according to guidelines of the National Committee for Clinical Laboratory Standards.

Statistical analysis. Data were compared by the 2-tailed χ^2 or Fisher's exact tests. Continuous variables were compared by the Wilcoxon rank sum test. EAggEC genotype and phenotype and demographic and clinical variables were correlated with diarrhea by using multivariate logistic regression models (version 6.0 software; Stata, College Station, TX).

Results

Study participants. We enrolled 111 patients with diarrhea and 68 asymptomatic control patients who were equivalent at baseline for demographic variables and HIV disease stage (table 1).

Intestinal pathogens. Enteric pathogens were detected among 31% of patients with diarrhea (table 1). Neither ileocolonoscopy of 17 patients nor esophagogastroduodenoscopy of 18 of 46 patients with chronic diarrhea revealed additional pathogens, except in 1 patient with dual intestinal infection due to *Leishmania* organisms and cytomegalovirus.

EAggEC PCR and adherence assay. *E. coli* was grown from 76 stool samples (68.5%) of 111 patients with diarrhea and from 51 stool samples (75%) of 68 control patients (table 2). The EAggEC cell adherence assay was positive in stool samples of 24 (22%) patients with diarrhea and in 21 (31%) control patients. EAggEC PCR was positive in 7 (6%) patients with diarrhea and in 1 asymptomatic control. Eight PCR-positive EAggEC were identified in specimens prepared from a swab of the MacConkey agar plate. When individual *E. coli* colonies were examined by PCR, EAggEC was detected in ⩾1 colony from 5 patients. Three of the PCR-positive isolates were cell–adherence assay negative.

EAggEC-associated clinical manifestations. One asymptomatic carrier of pCVD432 PCR-positive EAggEC (CD4 lymphocytes, 1028/mm^3) remained asymptomatic during follow-up. The

Table 1. Patient characteristics and intestinal organisms detected among human immunodeficiency virus (HIV)–infected patients with and without diarrhea.

Characteristics	With diarrhea[a]	Without diarrhea[a]
All patients	111 (100)	68 (100)
Women patients	11 (10)	1 (2)
Median age in years (range)	39 (25–79)	36 (27–61)
Mode of HIV acquisition		
Homosexual contact	75 (68)	45 (66)
Injection drug use	17 (15)	13 (19)
Heterosexual contact and other	19 (18)	10 (14)
No. with AIDS at baseline	40 (36)	20 (29)
Median CD4 lymphocytes/mm^3 (range)	188 (3–995)	259 (15–1028)
<50	16 (14)	7 (10)
50–200	42 (38)	17 (25)
>200	53 (48)	44 (65)
Median HIV-1 RNA (range), log^{10} copies/μL	3.0 (<2.6–6.4)	3.4 (<2.6–6.3)
Antiretroviral triple therapy	99 (89)	51 (75)
Pneumocystis carinii and *Toxoplasma gondii* chemoprophylaxis	46 (41)	18 (26)
No. with diarrhea without identifiable pathogen	77 (69)	64 (95)
No. of intestinal pathogens	40[b]	5[c]
Bacteria		
Enterovirulent *E. coli*		
EAggEC (PCR positive)[d]	7 (6)[e]	1 (1)[f]
Enteropathogenic *E. coli*	5 (5)	2 (3)
Enterotoxigenic *E. coli*	0	2 (3)
Verotoxin-producing *E. coli*	1 (1)	0
Clostridium difficile	6 (5)	ND
Campylobacter jejuni	3 (3)	ND
Salmonella species	2 (2)	ND
Aeromonas species	1 (1)	ND
Plesiomonas species	1 (1)	ND
Shigella species	0	ND
Protozoa		
Giardia lamblia	6 (5)	ND
Enterocytozoon bieneusi	3 (3)	ND
Cryptosporidia species	2 (2)	ND
Entamoeba histolytica	1 (1)	ND
Leishmania[g]	1 (1)	ND
Cytomegalovirus[g]	1 (1)	ND

NOTE. Data are no. (%) unless otherwise indicated. EAggE, enteroaggregative *Escherichia coli;* ND, not done; PCR, polymerase chain reaction.

[a] No statistical significant difference between groups with and without diarrhea with regard to all variables.

[b] Forty pathogens identified in 33 patients (6 patients with dual intestinal infection).

[c] Five pathogens identified in 4 patients (1 patient with dual infection).

[d] Difference between groups, $P = .16$.

[e] Includes 2 patients with coinfections due to EAggEC and enteropathogenic *E. coli* or *Enterocytozoon bieneusi,* respectively.

[f] Double infection due to EAggEC and enteropathogenic *E. coli.*

[g] Detected in intestinal biopsies.

Table 2. Detection of enteroaggregative *Escherichia coli* (EAggEC) in stool samples by using pCVD432 polymerase chain reaction (PCR) and cell adherence assay.

Patient group, specimens for EaggEC PCR	Total	*E. coli* culture positive	EAggEC adherence assay positive			EAggEC adherence assay negative		
			PCR positive	PCR negative	Total	PCR positive	PCR negative	Total
With diarrhea								
MacConkey agar plate	111 (100)	76 (68)						
Swab			4 (4)	20 (18)	24 (22)	3 (3)	49 (44)	52 (47)
Individual *E. coli* colonies			3 (3)[a]	21 (19)	24 (22)	2 (2)[b]	50 (45)	52 (47)
Without diarrhea								
MacConkey agar plate	68 (100)	51 (75)						
Swab			0	21 (31)	21 (31)	1 (1)	29 (43)	30 (44)
Individual *E. coli* colonies			0	21 (31)	21 (31)	ND[c]	30 (44)	30 (44)

NOTE. Data are no. (%) of patients. ND, not determined.

[a] Identical with 3 of 4 patients with EAggEC PCR–positive swab samples.

[b] Identical with 2 of 3 patients with EAggEC PCR–positive swab samples.

[c] EAggEC adherence assay was not done, because none of the 4 bacterial colonies obtained from MacConkey agar plate was biochemically found to be *E. coli.*

7 patients with diarrhea and EAggEC PCR-positive stool samples presented with nonspecific symptoms, including watery diarrhea (86%), abdominal pain (43%), and low-grade fever (57%). Bloody diarrhea was not reported. Hematology or serum chemistry did not reveal any abnormalities associated with EAggEC infection.

The following patient groups with diarrhea were compared with regard to clinical presentation: EAggEC PCR positive, PCR negative, EAggEC adherence assay positive, adherence assay negative, other intestinal pathogens, and no pathogen detected. No differences were found, except that patients with pCVD432 PCR-positive stool samples more frequently complained of fever. CD4 lymphocyte counts of patients with diarrhea and PCR-positive EAggEC (median, 141/mm^3; range: 3–995/mm^3), adherence-positive isolates (median, 264 mm^3; range, 3–842/mm^3), or other intestinal bacterial infections (median, 179/mm^3; range, 6–843/mm^3) were similar but significantly higher than in patients with enteric opportunistic infections (median, 17/mm^3; range, 3–83/mm^3).

The median follow-up was 20 months (range, 5–34 months). Of the 7 patients with PCR-positive EAggEC and diarrhea at baseline, 4 continued to have chronic persistent or chronic intermittent diarrhea, whereas diarrhea stopped during follow-up among 3 patients. All patients were receiving antiretroviral therapy, including a protease inhibitor, throughout the follow-up period. We observed no clear response to antibiotic treatment, although diarrhea improved or ceased in most patients during follow-up.

Correlation of EAggEC and diarrhea. Multivariate analysis showed a correlation between antiretroviral therapy and diarrhea ($P = .02$) and an association between the pCVD432 genotype and diarrhea ($P = .078$). The result of the adherence assay, demographic variables, CD4 lymphocytes, HIV-1 RNA, and chemoprophylaxis were not predictive for diarrhea.

Antibiotic resistance of pCVD432-positive EAggEC. Susceptibility testing of 13 different antibiotics showed resistance to trimethoprim-sulfamethoxazole (71%), ampicillin (71%), and cefalotin (43%). There was intermediate resistance to amoxicillin plus clavulanate (43%) and cefamandole (29%). One EAggEC PCR-positive isolate was resistant to ciprofloxacin.

Discussion

By using a PCR with primers complementary for the plasmid locus CVD432 [10], we detected EAggEC in 1 (1%) of 68 HIV-infected patients with and 7 (6%) of 111 without diarrhea, indicating a pathogenic potential of EAggEC in this patient group. The results of the EAggEC adherence assay, in contrast, did not correlate with diarrhea, which suggests that different EAggEC strains of heterogeneous virulence may exist. We could not identify risks for infection with EAggEC, such as source of drinking water, food, contact with persons with diarrhea, pets, or travel. As reported elsewhere [6], we also found a high rate of resistance to ampicillin, trimethoprim-sulfamethoxazole, and other antibiotics, which may be a consequence of trimethoprim-sulfamethoxazole chemoprophylaxis. A link between antibiotic resistance and virulence of EAggEC strains has been suggested [14].

Although EAggEC PCR-positive *E. coli* was the most prevalent intestinal organism associated with diarrhea, we were unable to definitively determine a causal relationship between its presence and diarrhea because of the low number of patients evaluated, and because most patients were on antiretroviral therapy, including protease inhibitors, which are associated with persistent diarrhea. Indeed, by multivariate analysis, we found antiretroviral therapy to be the strongest predictor of diarrhea.

EAggEC have been defined phenotypically by their property to aggregate in the HEp-2 or HeLa tissue culture adherence assay. Different DNA probes to identify *E. coli* carrying potential virulence traits that encode for adherence have been developed, but none of the factors has been consistently associated with the results of the adherence assay, clinical variables (e.g., diarrhea), or has been detected in most EAggEC strains from diverse geographic areas. Thus, the laborious and demanding cell adherence

assay remained the reference standard for identification of EAggEC. However, phylogenetic analyses of EAggEC indicate that phenotypically characterized EAggEC include genotypically different, presumably pathogenic and nonpathogenic organisms. Furthermore, clinical studies show that nonpathogenic EAggEC and non-EAggEC *E. coli* strains may exhibit the aggregative adherence pattern [2, 4].

The DNA fragment probe (CVD432) derived from the aggregative adherence plasmids of *E. coli* strains 17-2 and 042 is highly specific in detecting EAggEC [9]. Subsequently, a sensitive and specific PCR assay that uses primers complementary for pCVD432 was developed [10]. This assay identified 86% of reference EAggEC strains positive by the HEp-2 cell adhesion assay and was positive in only 0.96% of other diarrheagenic *E. coli.* However, some CVD432 probe–negative strains also carry an aggregative adherence plasmid, and the association of intestinal illness and CVD432 PCR–positive strains has not consistently been established. For example, Okeke et al. [2] found that only 26% of EAggEC obtained from Nigerian children hybridized with the CVD432 probe. Also, the prevalence of 8 adherence-encoding, plasmid-derived gene probes and 2 chromosomal gene probes did not differ between case and control patients, except that the aggregative adherence fimbrial subunit (AAF/II) was detected more frequently in EAggEC isolated from Nigerian children with diarrhea [2]. The EAST/1 heat-stable toxin may be linked to enterotoxicity for EAggEC, enteropathogenic *E. coli,* and enterohemorrhagic *E. coli* but cannot be used for specific screening for EAggEC [15].

Epidemiologic studies of EAggEC in western Europe that used pCVD432 PCR found a prevalence of 2% in German infants with diarrhea [3] and 6.4% among Austrian children and adults with diarrhea [16], but, to the best of our knowledge, no data on probe-positive *E. coli* among HIV-infected patients are available [3]. In a previous investigation on EAggEC, including cohorts of HIV-infected patients in Boston and Zurich, the HeLa cell assay demonstrated adherence in *E. coli* samples of 44% of patients with and 30% without diarrhea ($P = .05$) [6]. The CD4 lymphocyte counts of patients with EAggEC and diarrhea were significantly lower than among those without diarrhea ($P = .02$), which suggests that EAggEC may represent an opportunistic pathogen. In the present study, we could not duplicate these results using the same HeLa cell adherence assay. Furthermore, we found no significant differences with regard to CD4 cell count among patients with adherence-positive *E. coli,* associated with or without diarrhea, respectively, and adherence-negative *E. coli.*

EAggEC may be a clinically relevant pathogen that causes nonspecific intestinal disease, including acute or persistent watery diarrhea, abdominal pain, and often fever, not only among severely immunodeficient patients but also among HIV-infected persons whose cellular immunity was improved during antiretroviral therapy. Unfortunately, no widely accepted routine diagnostic technique is available that reliably detects all pathogenic EAggEC. Our data suggest that the pCVD432 PCR, possibly in combination with other probes including AAF/II [2], may be useful for diagnostic evaluation of HIV-infected adults with diarrhea, although such probes may miss a fraction of EAggEC. Further studies are required to assess the role of antibiotic treatment and the clinical impact of the high rate of antibiotic resistance of EAggEC strains.

Swiss HIV Cohort Study Members

The centers (members) of the Swiss HIV Cohort Study are as follows: University Hospital Basel (M. Battegay [Chairman of the Scientific Board], H. Bucher, P. Erb, Th Klimkait, and C. Rudin [Chairman of the Mother and Child Substudy]); University Hospital Bern (H. J. Furrer, M. Gorgievski, and W. Pichler); University Hospital Geneva (B. Hirschel, L. Perrin, and V. Schiffer); University Hospital Lausanne (Ph. Bürgisser, M. Egger, P. Francioli [President of the SHCS], F. Paccaud, G. Pantaleo, M. Rickenbach [Head of Data Center], P. Sudre, and A. Telenti); Ospedale Civico Lugano (E. Bernasconi and J. C. Piffaretti); Cantonal Hospital St. Gallen (R. Amiet, W. Fierz, C. Kind, and P. Vernazza); and University Hospital Zurich (M. Flepp [Chairman of the Clinical and Laboratory Committee], H. Günthard, P. Grob, B. Ledergerber, U. Lauper, M. Opravil, J. Schupbach, and R. Weber).

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